Impacts of antibiotic reagents on morphology and differentiation in Phaseolus vulgaris callus tissue

Ananya Gupta[1]

[1]Notre Dame High School

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Ananya Gupta
Notre Dame High School, San Jose, CA, USA

Abstract

Though one of the most common methods for reproducing plants, propagation through seeds is often not feasible because of a consistently low germination percentage. Instead, this project aims to study the viability of using *Phaseolus vulgaris* (known as the common bean) as a model organism in antibiotic resistance studies. Specifically, this project seeks to investigate the effectiveness of external antibiotics in promoting the growth and differentiation of common bean callus growth.

The single experimental group encapsulates bean callus growth medium with added cefotaxime, streptomycin, and kanamycin which are grown in standard growth medium with the addition of these antibiotics. The control group compares green bean callus growth in standard medium. To further evaluate the morphological differences between various mediums, a measure of the dry mass of the callus along with the study of its mitotic index was used to determine the effectiveness of each antibiotic reagent in improving the growth of the callus.

Ultimately, the results refute the original hypothesis which predicted that all four antibiotics would have a positive benefit on growth and regeneration of the callus tissue. Rather, when measuring callus health, only kanamycin had a significant effect (Mann-Whitney U = 3.5, p-value = 0.0385) on the growth factors of the callus tissue through its high mitotic index. Future research may apply these findings to focus on computational aspects in studying the effect kanamycin has on somatic embryogenesis via callus growth in an effort to inhibit bacterial

growth which reduces the chance of infection in the callus. By utilizing kanamycin resistance as a selectable marker, researchers can easily identify and select transformed plants taken up by foreign DNA and further simplify the study of genetically modified plant species, which has significant implications for the future of agricultural production.

Keywords: *Phaseolus vulgaris* callus, differentiation, mitotic index, Mann-Whitney U test

I. Introduction

With the global population expected to reach 9.8 billion by 2050 (United Nations 2017), there is a pressing need to increase crop yields and food production, while also addressing the challenges of climate change and environmental degradation. Green bean callus has long been a crucial area of study in plant biology, as it provides a unique model system to study the genetic and epigenetic mechanisms underlying plant growth and development in response to environmental stresses. The term "callus" was coined due to its resemblance to a wound-healing plant tissue (Neely, 1979, and references therein). When grown under specific environmental conditions and in controlling certain anthropogenic factors, somatic plant cells have the ability to integrate into specialized plant tissues without the need to be physically planted into soil. This experimental system has been used to study the molecular basis of cell proliferation, conveniently in vitro.

Many callus cells are totipotent and are able to regenerate the entirety of the plant body, which can be highly useful in studying macroscopic properties and undergo a process of generating new embryos from matured calli, known as somatic embryogenesis. (Steward et al., 1958; Nagata and Takebe, 1971). Somatic embryogenesis is a process in which somatic cells (non-reproductive cells) are induced to form structures resembling embryos, which can develop into new plants. The process involves the reprogramming of somatic cells to a totipotent state,

where they have the ability to differentiate into any cell type in the plant. The mitotic index can be used as an indicator of the cell proliferation rate during somatic embryogenesis. In particular, a high mitotic index can indicate that the cells are actively dividing and proliferating, which is a necessary step for the formation and growth of the somatic embryos. During the early stages of somatic embryogenesis, cells undergo several rounds of cell division, which can be monitored using the mitotic index. As the somatic embryos develop and mature, the mitotic index may decrease as the cells differentiate into specific cell types and the rate of cell division slows down. Therefore, the mitotic index can be a useful tool for optimizing somatic embryogenesis protocols, by determining the optimal conditions for promoting cell division and proliferation, and monitoring the progress of embryo development.

This groundbreaking discovery that is able to determine the various states of differentiation and dedifferentiation (Skoog and Miller, 1957) has allowed callus to be widely utilized in research and industrial applications (George and Sherrington, 1984; Bourgaud et al., 2001). However, the knowledge of the molecular mechanisms of partial organ regeneration within the callus have been limited due to the likelihood of secondary infections within the callus. When exposed to biotic and abiotic stress in changing environments over long periods of time, the callus tissue can be prone to these infections which can deter the growth and further morphogenesis (Du et al., 2019). This experiment is to ensure that the callus can grow healthily without common infections that prevent its growth and, as a result, prevent us from accurately evaluating its capabilities. This research project aims to evaluate the feasibility of using Phaseolus vulgaris (common bean) as a model organism for studying callus formation in vitro. While *Arabidopsis thaliana* has long been regarded as the standard model organism for plant biology, its extensive evaluation warrants the investigation of alternative model organisms. By

studying the regeneration of common bean callus in vitro, this project seeks to shed light on the epigenetic pathways that govern callus formation and optimize its growth conditions. Because of their high regenerative capacity and relatively fast callus formation, observing callus growth in the common bean makes them a convenient and efficient model for studying tissue culture and regeneration processes. One aspect involves testing the efficacy of external antibiotics integrated within the callus growth medium to maximize callus growth and minimize infections. This is an important consideration as callus growth can be impeded by common infections that prevent its growth, hindering accurate evaluation of its capabilities.

II. Materials and Methods

2.1 Regeneration Media & Antibiotic Treatment

Media is prepared using deionized water, with added D-sucrose, Ultra-Pure (PhytoTech Labs), agarose (Sigma-Aldrich), and carrot callus initiation basal medium (PhytoTech Labs, C212). Initiation media provides necessary hormonal balance and nutrient support for the induction and development of callus tissue from bean explants. A 1M potassium hydroxide (Carolina Labs) additive is pipetted to maintain pH. Added to the callus growth medium, potassium hydroxide acts as a pH regulator, helping to maintain the optimal pH for callus growth by neutralizing any acidic components in the medium and preventing pH fluctuations that could hinder callus development. Media is sterilized by autoclaving at 165° C and 1.05 kg/cm^2 (15-20 psi). Once medium is out of autoclave, it is cooled until it is at room temperature. Antibiotics are added respectively at a 5% concentration after the media has cooled to prevent further degradation. 4 iterations of media are prepared for the respective antibiotics and a control group. Prepared media is poured into Petri dishes, filling them halfway.

Cefotaxime (PhytoTech Labs, C380), kanamycin monosulfate (P212121), and streptomycin sulfate salt (Sigma-Aldrich) are utilized in various media preparations. These reagents serve as selective compounds that can inhibit growth of infectious microorganisms. These antibiotics have not yet been extensively evaluated in *P. vulgaris* plant tissue culture and hence utilized to prevent contamination of bacteria and fungi, allowing the growth of healthy callus. Evaluating these common antibiotics is crucial in maintaining the integrity and reproducibility of the experiments, ensuring the purity of callus cultures by eliminating unwanted microbial contamination. Understanding the response of green bean callus to these antibiotics is insightful in developing efficient and reliable protocols for plant propagation and genetic engineering.

2.2 Plant Material Sterilization

Store bought green beans were refrigerated at 2° C under cool white fluorescent light (20-hr photoperiod, 50-90 μmol $m^{-2}s^{-1}$). Beans were then extracted by hand and placed in an empty Petri dish. Isopropanol (Sigma-Aldrich) is poured into the Petri dish containing the beans, filling about halfway, soaked for up to three minutes, and strained. Beans are added to a beaker containing a diluted bleach solution (sodium hypochlorite, 5% concentration, Carolina Labs), soaked for another 15 minutes, and strained. Then, beans are rinsed in lukewarm deionized water (37.2° C) three times. Using a tweezer, callus funiculi are extracted from each bean. 3-4 extracted funiculi are placed per plate. Plates are parafilmed and stored in a dark and cool place (22° C).

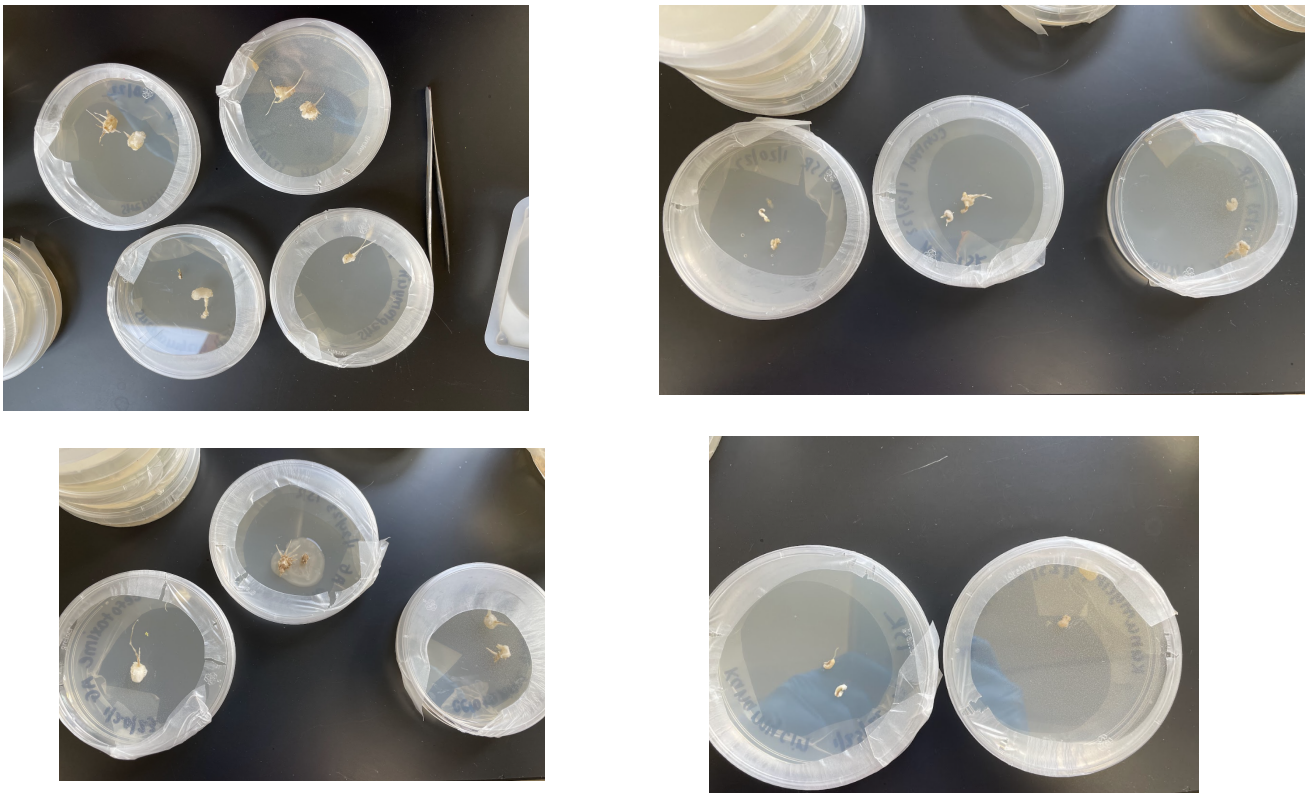


Figure 1.1-1.4. Callus growth after a week in observance

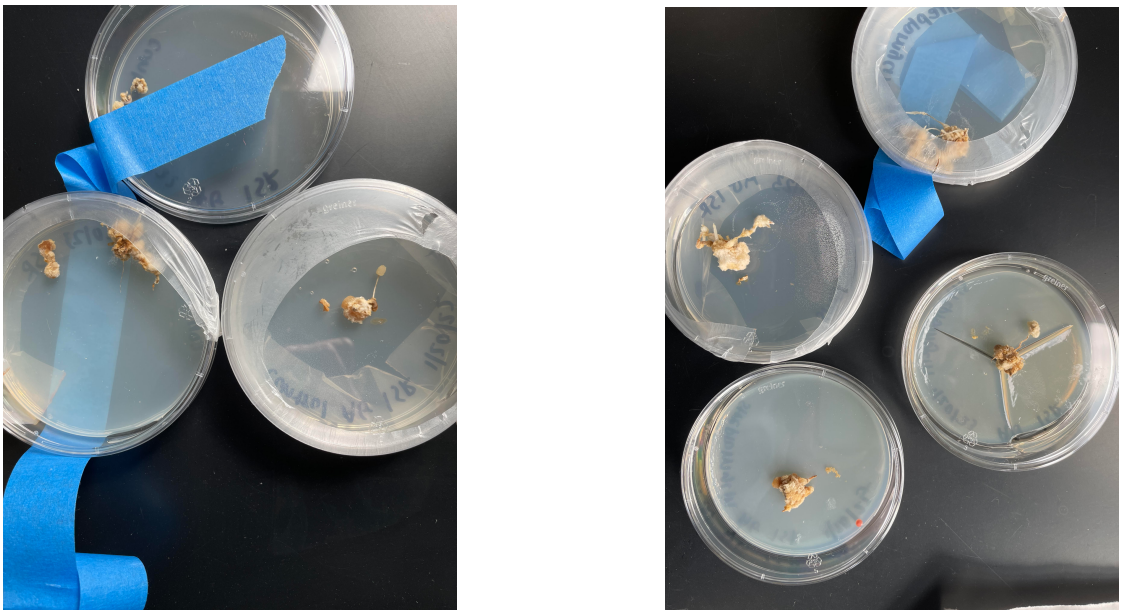


Figure 2.1-2.2. Callus growth after two weeks

2.3 Data Collection and Analysis

For each experimental group and single control group, ten petri dishes were used per treatment, each containing three to four bean funiculi (n = 100). Using regeneration medium with necessary initiation nutrients, the effects of theses external antibiotic reagents was integrated within the callus growth medium to observe if they work to improve the differentiation and growth of *P. vulgaris* callus growth and if a certain reagent works particularly well in improving morphological differentiation. Two sets of the experiment were carried out, both measured over a span of two weeks, in measuring the dry mass of the callus as well as its changing mitotic index of each antibiotic and control group. Growth in the second group was significantly better because each antibiotic was added to the medium after the preparation had been autoclaved and cooled, to prevent any degradation of the antibiotic, reducing its purpose and effect. High temperatures denature and degrade antibiotic molecules, reducing its effectiveness in minimizing unwanted infections. By waiting for the medium to cool, antibiotics can be added under conditions that preserve their stability, ensure their full potency and ability to inhibit bacterial growth in the culture.

The dry mass of the callus was measured at the end of the two week growth period after putting the callus in a food dehydrator (Colzer) to completely remove any moisture that may add unwanted mass to the samples. Data was further compared to record morphological differences between the experimental groups and the effects of the media. It is an effective metric, providing quantitative information about the accumulation of biomass and can indicate effectiveness of growth conditions or treatment. Measurements of the dry mass represent the amount of accumulated tissue, including cells, extracellular matrix, and other organic components. This data point provides information on the overall productivity of the culture and can be used to

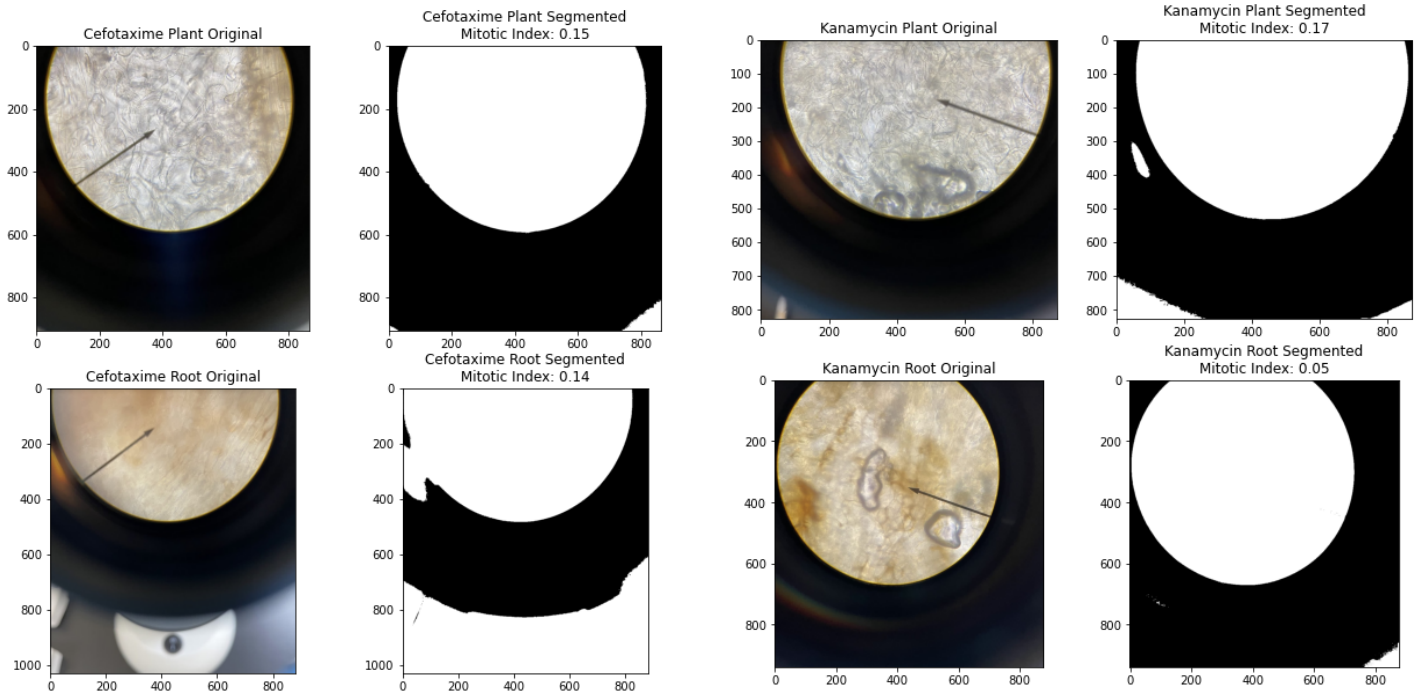
compare different experimental conditions, in this case, the effects of external antibiotics. By tracking changes in dry mass and morphological differences, one is able to evaluate the effects of various factors on callus growth, such as different nutrient formulations or genetic changes. It provides a standardized measurement in evaluating growth. Other qualitative parameters, such as visual observations or fresh weight measurements, can be influenced by factors like water content or morphological variations. Dry mass eliminates the influence of water content and provides a consistent measurement that can be compared within the experimental and control groups.

A nucleic staining procedure was performed to measure the changing mitotic index between the growth of the callus for each experimental and control group. To begin, one preserved growth callus specimen was isolated in a weight boat. A single drop (50μM) of N/10 hydrochloric acid solution (Fisher Scientific) was added, followed by two to three drops (100-150 μM) of laboratory grade aceto-carmine (Schneider, Carolina Labs), a staining solution known for its ability to specifically highlight chromosomes within cells and facilitate the visualization of the mitotic process. A small portion (2-3 mm) of callus root and plant tip was excised, and carefully placed on separate microscopic slides. A drop of water was placed on the slide, and a cover tip was mounted over the sample. The prepared slide was examined using a monocular biological microscope (Open Box 40X - 1000X Monocular Biological Microscope) at a x550 magnification. This procedure was repeated every four days to observe active growth over time. The mitotic index is a useful metric when measuring plant growth as it is able to provide a quantification of cell division. A high mitotic index indicates a higher proportion of dividing cells, which suggest active growth and regeneration of the tissue. The mitotic index provides information about the growth dynamics of the callus, and monitoring changes in the

mitotic index over time can help evaluate the effectiveness of different growth conditions or treatments on cell proliferation. The mitotic index provided data on developmental stage determination, in tracking temporal progression of callus growth and development. It is an indicator of the health and quality of callus culture. A low or declining mitotic index may suggest tissues with cell viability, nutrient availability, or other factors affecting cell division. Considering this change over time can help identify suboptimal conditions or stressors that might hinder callus growth.

III. Results

A data set of 500 images was compiled through the staining procedure with an average of 125 images per experimental and control group over the two weeks. Anaconda was used to write various programs that were able to process the data and output images that gave an overall mitotic index or rate of cell proliferation over time. One program was written to calculate the mitotic index for every plant and display the cell image with the average mitotic index. Each antibiotic's individual data set was given with the plant and root growth. The code was able to threshold the image and calculate the rate of division.



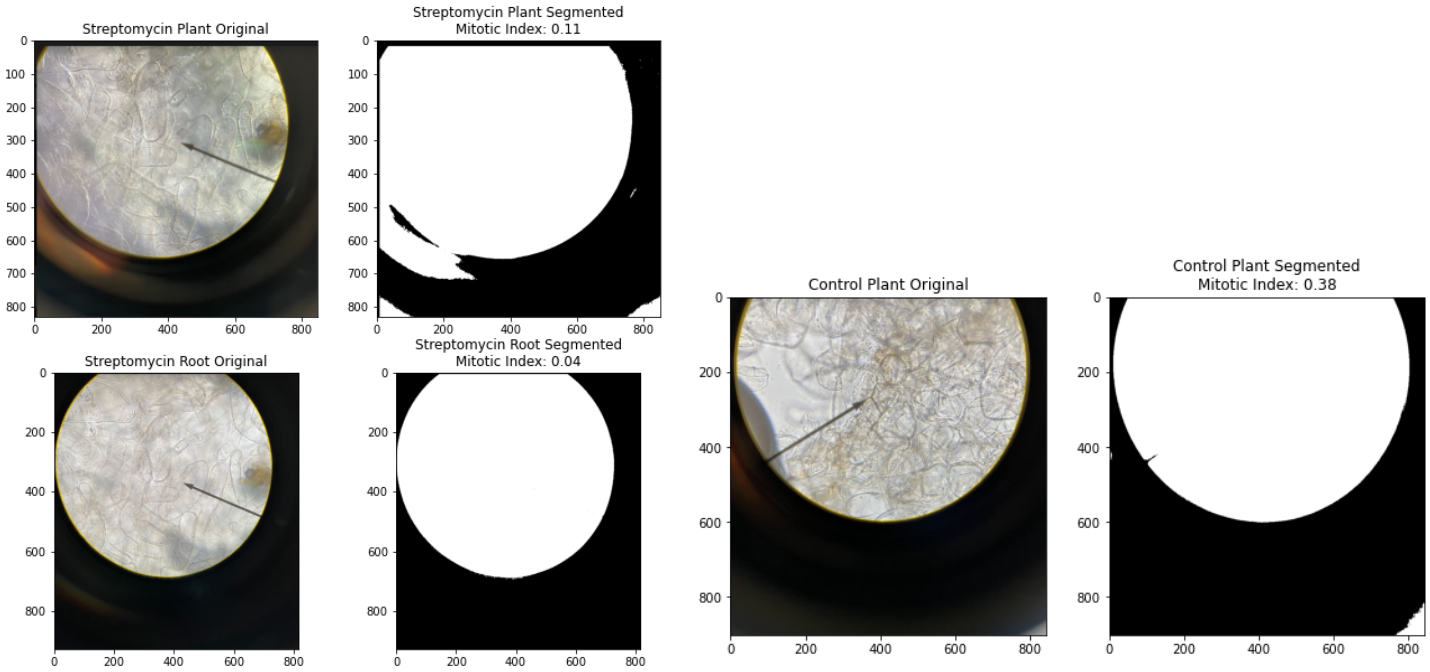


Figure 3.1-3.4. Calculated mitotic index for each antibiotic group with plant and root growth. Control did not have any root growth, hence, only plant growth is shown in the images above. Standard sample size, by group: 5.

Another program was written to compare between the root growth dataset of every antibiotic implemented and compared between the plant growth. A bar graph was displayed with an error bar to indicate the relation of the p-value and significance. It uses the OpenCV library to read and process the images, converting them to grayscale and thresholding them to separate the nuclei from the background. It then uses contour detection to identify the individual nuclei and calculates the mitotic index by counting the number of nuclei in mitosis (determined by their size) and dividing by the total number of nuclei. The code uses a dictionary to store the image paths for each antibiotic treatment, and loops through each treatment to analyze the corresponding root and plant images. It then calculates the mitotic indices and standard errors for each group, and stores them in separate lists. A final output is a bar graph using the Matplotlib library, with error bars calculated from the standard errors of the mean. It provides a useful tool

for analyzing the effects of different antibiotic treatments on cell division in plants, and could be modified or extended for other applications involving image analysis and quantitative measurements.

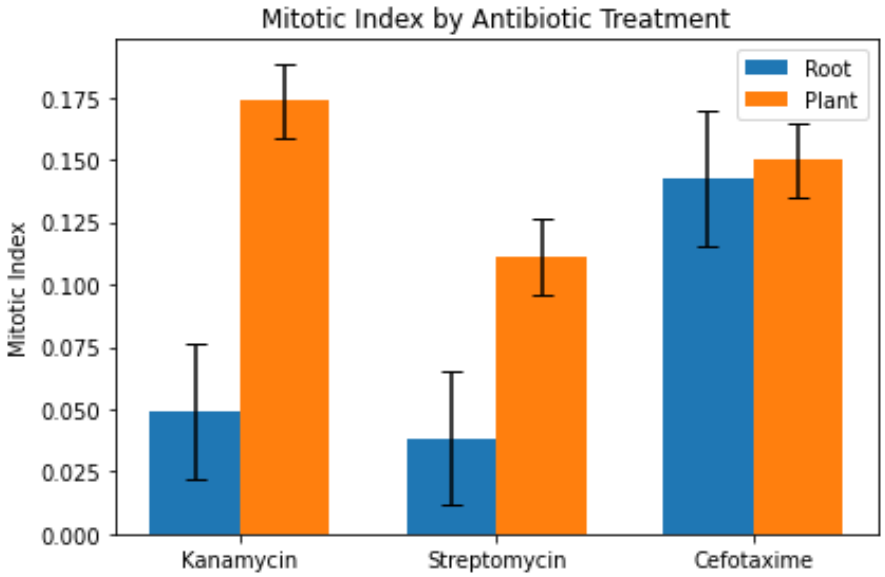


Figure 4. Comparison with standard error values

A third program was written to threshold the images using two distinct mechanisms: Otsu and Sobel edge detection. The program defines a dictionary called “antibiotics” containing the names of the three antibiotics as keys, with each corresponding root and plant image stored as values. It is able to loop through each antibiotic in the dictionary and for each image, it reads the root and plant images in grayscale using OpenCV’s “cv2.imread()” method. It applies Otsu thresholding on the plant and root images using openCV’s “cv2.threshold()” method with the "cv2.THRESH_BINARY+cv2.THRESH_OTSU" flags. This thresholding technique automatically calculates a threshold value that separates the foreground and background regions in the image. It also applies Sobel edge detection on each root and plant images using OpenCV’s "cv2.Sobel()" method with a kernel size of 5. This technique detects edges in an image by

calculating the gradient of the image intensity at each pixel. In the output, it displays the original root and plant images, as well as the Otsu thresholded, Sobel detected, and HSV converted versions of both images using the Matplotlib library's "pyplot.subplots()" and "imshow()" methods. The images are displayed in a 2x4 grid with the title of the antibiotic as the main title of the plot.

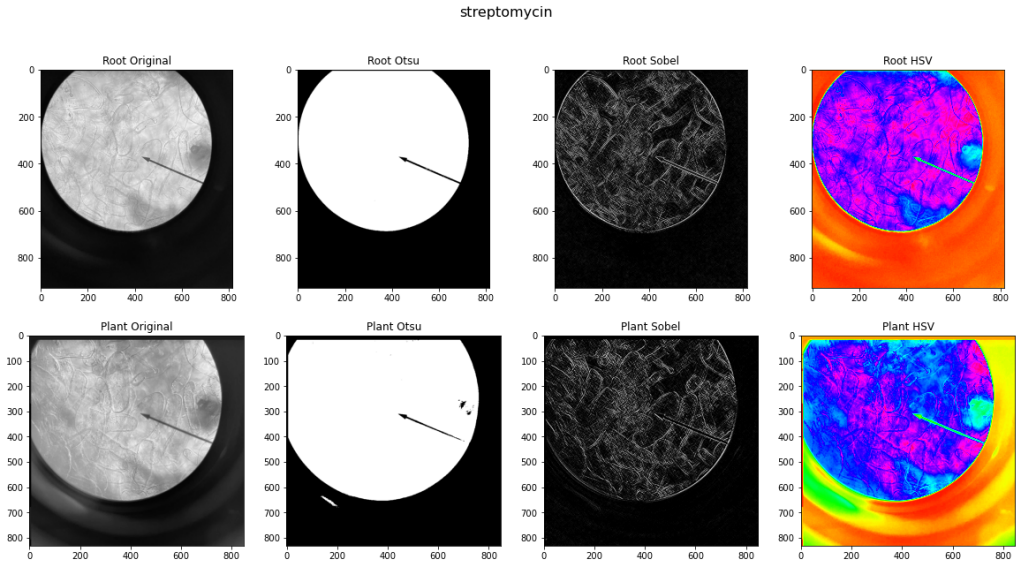


Figure 5.1 Otsu, Sobel, and HSV thresholding on the streptomycin group

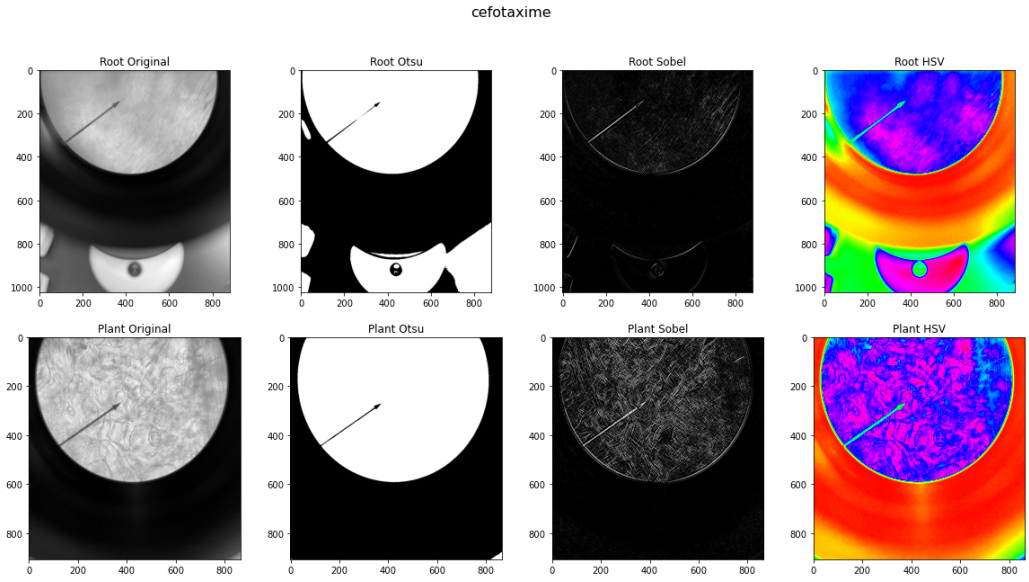


Figure 5.2 Otsu, Sobel, and HSV thresholding on the cefotaxime group

A fourth program performed two analyses in measuring significance of the data. The first part of the code calculates the Mann-Whitney U statistic and the two-tailed p-value for the experimental groups. The Mann-Whitney U test is a non-parametric statistical test used to compare two independent groups and determine if there is a significant difference between them. The second part of the code loads images of the plant and root samples treated with different antibiotics as well as the control group. The pixel intensities of the images are extracted and plotted using box plots, with each antibiotic treatment as well as their interquartile range and the mean. The purpose of the box plots is to compare the mitotic index data between the different antibiotic treatments and the control and to visualize any differences in the changing mitotic index between the two groups. A higher mitotic index indicates a higher rate of cell division, which means that the callus tissue is actively growing and proliferating, indicated most strongly through kanamycin. The Mann-Whitney U test was performed, which showed no statistically significant difference in dry mass between the experimental and control groups (U=11.0, p-value = 0.114). However, the mitotic index was higher in the kanamycin group compared to the control group (U = 3.5, p-value = 0.0385).

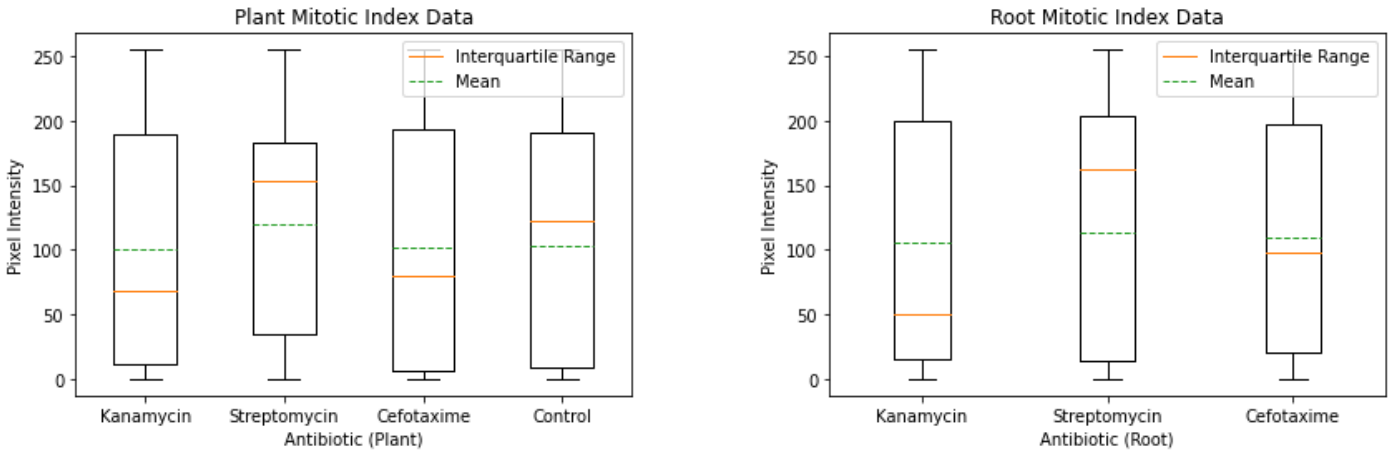


Figure 6.1-6.2. Plant and root mitotic index data between the two antibiotic group

Sample	Control	Kanamycin	Streptomycin	Cefotaxime
1	0.02g	0.06g	0.03g	0.01g
2	0.02g	0.06g	0.02g	0.02g
3	*	0.04g	0.03g	0.02g
4	*	0.05g	*	*
5	*	0.04g	*	*

Figure 7. Callus dry mass over growth period; * Indicates that callus tissue was infected with external bacterial growth

IV. Discussion

Through its high mitotic index, the study suggests that kanamycin is most effective. It can be concluded that it is actively proliferating, indicating positive chromosomal health and active growth.

4.1 Selectivity

Kanamycin makes for an effective selectable marker agent for screening modified cells. Cefotaxime and streptomycin are broad spectrum antibiotics that can kill both contaminated bacteria and plant cells that do not contain a resistance marker. When performing genetic transformation experiments, researchers often introduce a resistance gene, such as the nptII gene, into the plant cells along with the gene of interest. This resistance gene confers resistance to kanamycin, allowing transformed cells to survive and grow in the presence of the antibiotic. This way, kanamycin serves as a selective agent to identify and screen for successfully transformed cells. In contrast, broad-spectrum antibiotics like cefotaxime and streptomycin can be effective against a wide range of bacteria, but they lack the specificity of kanamycin. These antibiotics can kill both contaminated bacteria and plant cells that do not contain a resistance marker, leading to

a higher risk of undesired cell death and reduced efficiency in selecting for transformed cells. Cefotaxime, for instance, is a broad-spectrum cephalosporin antibiotic that acts against many bacteria by inhibiting cell wall synthesis. However, it is not selectively toxic to bacteria and can affect plant cells as well. Streptomycin, another broad-spectrum antibiotic, targets the bacterial ribosomes, disrupting protein synthesis. However, it can also impact the ribosomes in plant cells, leading to potential cytotoxic effects. The lack of selectivity of cefotaxime and streptomycin makes them less ideal for use as selectable markers in plant tissue culture experiments. Their broad spectrum of activity increases the risk of non-specific toxicity and can result in the loss of plant cells that do not contain the desired resistance marker. Kanamycin's specificity for targeting prokaryotic cells, coupled with its minimal impact on plant cells carrying the resistance marker, makes it a preferred choice as a selectable marker agent. In contrast, cefotaxime and streptomycin's lack of specificity and potential toxicity to plant cells without resistance markers make them less suitable for efficient screening of genetically modified cells in plant tissue culture.

4.2 Mode of Action

Kanamycin belongs to the aminoglycoside class of antibiotics, which target the bacterial ribosome and inhibit protein synthesis. This class of antibiotics binds to the 30S subunit of the bacterial ribosome, leading to misreading of the mRNA code and the production of non-functional or truncated proteins. By inhibiting protein synthesis, kanamycin effectively kills or inhibits the growth of susceptible bacterial cells. In plant tissue culture, kanamycin is often used as a selectable marker agent to promote the growth of genetically modified cells carrying a resistance gene, such as the nptII gene. The presence of this resistance gene allows the transformed plant cells to survive and proliferate in the presence of kanamycin. Meanwhile,

non-transformed cells lacking the resistance gene will be inhibited or killed by kanamycin, providing a selective advantage for the desired transformed cells. On the other hand, cefotaxime and streptomycin have different modes of action that may not be as effective in promoting callus growth: Cefotaxime belongs to the cephalosporin class of antibiotics and acts by inhibiting bacterial cell wall synthesis. It interferes with the formation of peptidoglycan, a crucial component of the bacterial cell wall, leading to cell wall disruption and bacterial cell death. However, this mechanism of action may not directly contribute to promoting callus growth in plant tissue culture. In fact, the inhibition of cell wall synthesis may have adverse effects on the growth and development of plant cells as well. Streptomycin is an antibiotic that binds to the 30S subunit of the bacterial ribosome, similar to kanamycin. It inhibits protein synthesis by interfering with the accurate reading of the mRNA code, resulting in defective protein production and bacterial cell death. However, unlike kanamycin, streptomycin does not have the same selective advantage for genetically modified cells as it lacks the specific resistance gene associated with plant transformation. Consequently, streptomycin's mode of action may not be as effective in promoting callus growth in a selective manner. Kanamycin's mode of action, targeting the bacterial ribosome and inhibiting protein synthesis, makes it an effective selectable marker agent for promoting callus growth in plant tissue culture. In contrast, cefotaxime's inhibition of cell wall synthesis and streptomycin's non-specific action may not provide the same selective advantage and may have detrimental effects on plant cell growth. Cefotaxime and streptomycin have different modes of action (inhibition of cell wall synthesis and protein synthesis) which may not be as effective for promoting callus growth. Kanamycin can be concluded as the preferred antibiotic choice when aiming to promote callus growth in a selective manner during plant tissue culture experiments.

4.3 ROS Induction

Kanamycin has been shown to induce the production of reactive oxygen species (ROS) in plant cells which play an important role in callus growth and development and may act as signaling molecules that trigger cell division and differentiation. ROS, such as hydrogen peroxide (H2O2), superoxide radicals (O_2), and hydroxyl radicals (OH), are highly reactive molecules that function as signaling molecules in various physiological processes. In the context of callus growth, ROS have been implicated in regulating cell division, differentiation, and organogenesis. The controlled production of ROS can act as signaling molecules, triggering specific cellular responses and promoting cell proliferation and differentiation. ROS are involved in numerous cellular processes, including cell cycle progression, hormone signaling, defense responses, and oxidative stress adaptation. This can be attributed to the interference of kanamycin with the bacterial ribosomes, which can indirectly impact mitochondrial function and electron transport chains, leading to an imbalance in cellular redox homeostasis. The resulting increase in ROS levels can stimulate various signaling pathways and contribute to callus growth and development. In contrast, cefotaxime and streptomycin, with their different modes of action (inhibition of cell wall synthesis and protein synthesis, respectively), have not been reported to have the same stimulatory effect on ROS production in callus tissue. These antibiotics may not disrupt cellular processes in a manner that leads to the generation of ROS or the subsequent signaling events associated with ROS. The ability of kanamycin to induce ROS production in plant cells during callus growth can provide additional benefits beyond its role as a selectable marker. The ROS-mediated signaling pathways activated by kanamycin can influence cell division, differentiation, and other important cellular processes, potentially enhancing callus growth and development. In contrast, cefotaxime and streptomycin do not exhibit the same

stimulatory effect on ROS production in callus tissue. Cefotaxime and streptomycin have not been shown to have this same stimulatory effect on ROS production in callus tissue.

Future applications of bacterial resistance can be studied with a computational lens to further study the effect kanamycin has on somatic embryogenesis via callus growth to inhibit bacterial growth, reducing the chance of infection in the callus, further exploring the gene expression patterns in callus tissue in response to the different antibiotics used in this study. This could be done through techniques such as RNA sequencing or microarray analysis, to identify which genes are upregulated or downregulated in response to the antibiotics. This study is significant as by evaluating the effectiveness of these antibiotics, researchers can determine the optimal concentration and exposure time required to inhibit microbial growth effectively. This knowledge is crucial for maintaining uncontaminated callus cultures and ensuring the success of plant tissue culture experiments. Microbial contamination poses a significant challenge in plant tissue culture. Bacteria and fungi can hinder the growth and development of callus cultures, leading to unreliable results and experimental failure. Evaluating antibiotics allows researchers to select the most appropriate ones to prevent contamination, ensuring the integrity and purity of the cultures. While antibiotics are essential for controlling microbial contamination, they can also have detrimental effects on the growth and development of plant cells. By evaluating these antibiotics, researchers can identify the concentration threshold at which they start to negatively impact callus growth. This information is crucial for minimizing antibiotic-induced toxicity and optimizing culture conditions. Evaluating antibiotics in green bean callus growth helps establish standardized protocols for successful tissue culture. Researchers can determine the most effective antibiotic combinations, concentrations, and exposure durations, providing guidelines for consistent and reproducible results across different laboratories and experiments. Understanding

the response of green bean callus to these antibiotics has broader implications beyond the specific study. It can provide valuable insights into the potential use of these antibiotics in controlling microbial contamination in other plant tissue culture systems. This knowledge can aid in developing effective strategies to combat contamination issues in various plant species, thereby enhancing the reliability and scalability of plant propagation and genetic engineering techniques.

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